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RESEARCH PAPER

OXI1 protein kinase is required for plant immunity against *Pseudomonas syringae* in *Arabidopsis*

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Abstract

Expression of the *Arabidopsis* Oxidative Signal-Inducible1 (OXI1) serine/threonine protein kinase gene (At3g25250) is induced by oxidative stress. The kinase is required for root hair development and basal defence against the oomycete pathogen *Hyaloperonospora parasitica*, two separate H_2O_2-mediated processes. In this study, the role of OXI1 during pathogenesis was characterized further. Null *oxi1* mutants are more susceptible to both virulent and avirulent strains of the biotrophic bacterial pathogen *Pseudomonas syringae* compared with the wild type, indicating that OXI1 positively regulates both basal resistance triggered by the recognition of pathogen-associated molecular patterns, as well as effector-triggered immunity. The level of *OXI1* expression appears to be critical in mounting an appropriate defence response since *OXI1* overexpressor lines also display increased susceptibility to biotrophic pathogens. The induction of *OXI1* after *P. syringae* infection spatially and temporally correlates with the oxidative burst. Furthermore, induction is reduced in *atrbohD* mutants and after application of DPI (an inhibitor of NADPH oxidases) suggesting that reactive oxygen species produced through NADPH oxidases drives *OXI1* expression during this plant–pathogen interaction.

Key words: *Hyaloperonospora parasitica*, plant defence, *Pseudomonas syringae*, reactive oxygen species, signal transduction.

Introduction

Plant immunity to the wide variety of potential pathogens involves a complicated web of components ranging from preformed defence barriers to signalling molecules such as reactive oxygen species (ROS), protein kinases, and hormones to elicit appropriate end responses (Thomma *et al.*, 2001; Ingle *et al.*, 2006; Torres *et al.*, 2006). The current viewpoint is that there are two major branches of plant immunity as reviewed by Jones and Dangl (2006). The first encompasses a general immune response triggered by the recognition of evolutionary conserved pathogen-associated molecular patterns (PAMPs), for example, bacterial flagellin, lipopolysaccharides, and fungal chitin. This PAMP-triggered immunity (PTI) activates a series of inducible basal defence mechanisms such as callose deposition and defence gene expression and is successful against non-host pathogens. Virulent pathogens suppress PTI via pathogen effector molecules which can target components of the basal defence mechanism and induce effector triggered susceptibility (ETS). This enables virulent pathogens to cause disease on susceptible host plants (Jones and Dangl, 2006). The second layer of immunity occurs when the host plant harbours a resistance protein to detect either the presence and/or activity of one or more effectors resulting in the rapid activation of plant defence responses and disease resistance known as effector triggered immunity (ETI) (Mackey *et al.*, 2002; Jones and Dangl, 2006). Although ETI responds faster to pathogen infection, PTI and ETI share many regulatory components (Ingle *et al.*, 2006).

Central to plant immunity against biotrophic pathogens is the accumulation of ROS, which apart from direct functions in toxicity (Keppler *et al.*, 1989) and oxidative cross-linking of plant cell walls (Bradley *et al.*, 1992; Fry

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et al., 2000) serve a signalling role in mounting the defence response (Grant and Loake, 2000). A key feature of ROS signalling is regulation of the hypersensitive response (HR) characterized by rapid localized cell death at the infection site as well as the induction of defence-related genes (Levine *et al.*, 1994; Lamb and Dixon, 1997; Grant and Loake, 2000). Chemical inhibition of ROS accumulation following pathogen challenge in *Arabidopsis* led to a reduction in the HR and inhibited expression of the defence gene *glutathione-S-transferase1* (Alvarez *et al.*, 1998). Conversely, elevation of H_2O_2 levels either through suppression of antioxidant enzyme activity, such as in transgenic tobacco plants deficient in peroxisomal catalase activity (Chamnongpol *et al.*, 1998), or expression of enzymes required for ROS production, such as in transgenic potato plants expressing glucose oxidase (Wu *et al.*, 1997), resulted in a primed immune response with accumulation of salicylic acid (SA), expression of defence-related genes, and enhanced resistance to a broad range of pathogens. More recently, *Arabidopsis* ascorbate-deficient mutants were found to exhibit microlesions, constitutive *Pathogenesis Related (PR)* gene expression, and increased resistance to *Pseudomonas syringae* infection (Pavet *et al.*, 2005) providing further evidence for the role of ROS accumulation in disease-resistance responses.

Genetic evidence points to a role for the respiratory burst NADPH oxidase as the principal source of ROS production during pathogen challenge (Torres *et al.*, 2002). *Arabidopsis* mutants lacking either or both of the respiratory burst oxidase genes, *AtrbohD* and *AtrbohF*, which encode catalytic subunits of the NADPH oxidase, displayed a reduction in H_2O_2 accumulation and the HR in response to avirulent *P. syringae* pv. *tomato* DC3000 *avrRpm1* infection compared to wild-type *Arabidopsis* (Torres *et al.*, 2002). However, following challenge with a virulent *Hyaloperonospora parasitica* strain (formerly known as *Peronospora parasitica*; Constantinescu and Fatehi, 2002), the *atrbohF* mutant displayed an enhanced HR and increased resistance to this pathogen (Torres *et al.*, 2002) indicating that the HR is differentially regulated by ROS accumulation depending on the invading pathogen. Alternative mechanisms for ROS production during pathogen attack have been demonstrated, for example, pharmacological inhibition of peroxidase activity during pathogen treatment resulted in a significant decrease in *GST1* expression, a marker of ROS accumulation, compared to pathogen treatment alone (Grant *et al.*, 2000). More recently, overexpression of the pepper extracellular peroxidase *CaPO*$_2$ gene in *Arabidopsis* conferred enhanced disease resistance against *P. syringae* and increased H_2O_2 levels following infection (Choi *et al.*, 2007). The increased H_2O_2 production was sensitive to chemical inhibition of peroxidase activity but unaffected by inhibition of NADPH oxidase.

Despite the strong correlation between ROS accumulation and disease resistance, current understanding of the discriminators of ROS signalling is sorely limiting. The OXIDATIVE SIGNAL-INDUCIBLE1 (OXI1) protein kinase has emerged as a potential player linking ROS accumulation to disease resistance in response to virulent *H. parasitica* attack (Rentel *et al.*, 2004). *OXI1* is not only induced by the exogenous application of H_2O_2 and challenge with virulent *H. parasitica* Emco5 but the *oxi1* null mutant also displayed increased susceptibility compared to wild-type *Arabidopsis* following infection with Emco5 (Rentel *et al.*, 2004). Furthermore, OXI1 is required for the partial activation of MPK3 and MPK6 in response to treatment with H_2O_2 and cellulase, mimicking pathogen attack (Rentel *et al.*, 2004). Both MPK3 and MPK6 are involved in the mitogen-activated protein kinase cascade activated following recognition of bacterial flagellin by the receptor-like kinase FLS2 (Asai *et al.*, 2002) which initializes the induction of defence genes such as *WRKY22/29* and *GST* and is effective in defence responses against both bacterial and fungal pathogens (Gomez-Gomez *et al.*, 2001; Asai *et al.*, 2002; Chinchilla *et al.*, 2006). In this report, a role for *OXI1* in *Arabidopsis* is further extended to plant immunity against the bacterial pathogen *P. syringae* and NADPH-produced ROS is shown to drive expression of *OXI1* during this plant–pathogen interaction. Interestingly, regulation of *OXI1* expression levels appears important in mediating an appropriate defence response, since both down-regulation and overexpression of *OXI1* results in enhanced susceptibility to biotrophic pathogens.

Materials and methods

Plant growth conditions

Arabidopsis thaliana plants were grown on a 1:1 (v/v) soil mix composed of peat (Jiffy Products, International AS, Norway) and vermiculite in a controlled environment under a 16/8 h light/dark cycle at 21°C, 55% relative humidity, and fluorescent light of 80–100 μmol photon m^{-2} s^{-1}.

Plant lines

Wild-type *Arabidopsis* seeds were acquired from Lehle Seeds (Lehle, Texas, USA). The *oxi1* null mutant, *OXI1* complemented, and *OXI1::GUS* transgenic lines were the same as those used in Rentel *et al.* (2004). The *atrbohD* T-DNA mutant line used was that described in Torres *et al.* (2002).

Generation of 35S::OXI1 *and* 35S::OXI1-YFP *constructs*

A 1.4 kb DNA fragment of *OXI1* including the entire coding region and its intron was PCR amplified from genomic DNA from the Ws-2 ecotype with the primers 5′-GCGCCTGCAGGTCGACATTATGCTAGAGGG-3′ and 5′-GCGCGGATCCGTACACCATAGTCCATAGAC-3′. The 2.5 kb OXI1–YFP protein fusion comprising a 1.4 kb *OXI1* DNA fragment, a 1.1 kb *YFP* coding region, and a *c-myc* epitope tag, was PCR amplified from the pBluescript SK$^-$ plasmid harbouring the *OXI1-YFP-cmyc* construct (Rentel, 2002) with the primers 5′-GCGCGGATCCGTCGACATTATGCTAGAGGG-3′ and 5′-GCGCCCCGGGCAAGACCGGCAACAGGATTC-3′.

Both PCR products were cloned into the pUC2X35S plasmid containing two *35S CaMV* promoters with the restriction enzymes *Pst*I and *Bam*HI for *OXI1* and *Bam*HI and *Xma*I for *OXI1-YFP-cmyc*, respectively, followed by subcloning into the pBINPLUS binary vector through the unique restriction sites *Asc*I and *Pac*I. Both vectors were a gift from Malcolm Campbell (Department of Botany, University of Toronto, Canada). The resulting plasmids were transformed into the C58C1 strain of *Agrobacterium tumefaciens* and transformed into *Arabidopsis* plants of the Ws-2 ecotype by the floral dip method (Clough and Bent, 1998). 25 μg ml^{-1} kanamycin was used for selection of homozygous lines.

Pathogen infections

Inoculations with virulent *Pseudomonas syringae* pv. *tomato* DC3000 and avirulent *P. syringae* harbouring the *avrB* gene were performed as described in Murray *et al.* (2002). The avirulent strain was maintained and grown on King's broth media (King *et al.*, 1954) supplemented with 50 μg ml^{-1} rifampicin and 50 μg ml^{-1} kanamycin. Inoculation and assessment of *Hyaloperonospora parasitica* sporulation was determined as described in Rentel *et al.* (2004). All pathogen infection experiments were repeated at least three times.

In vivo *histochemical GUS and DAB staining*

GUS staining of *Arabidopsis* leaves was performed as previously described by Rentel *et al.* (2004). The presence of H_2O_2 was detected by gently shaking leaves submerged in a 1 mg ml^{-1} 3,3′-diaminobenzidine (DAB) solution for 2–3 h at room temperature until a reddish-brown precipitate was observed. Images for both GUS and DAB staining were obtained by scanning the leaves with a Canonscan 8400F Scanner.

Northern blot analysis

Total RNA was extracted using either the RNeasy Plant Total RNA kit (Qiagen, UK) as per the manufacturer's instructions or a guanidinium thiocyanate-phenol-chloroform extraction protocol (Chomczynski and Sacchi, 1987). Electrophoresis and transfer of RNA onto nylon membrane was performed as previously described by Murray *et al.* (2007). DNA probes were labelled with ^{32}P using a Megaprime DNA labelling kit (Amersham, UK) and hybridized to total RNA in hybridization buffer composed of 5× SSC, 50% (v/v) formamide, 0.5% (v/v) SDS, 5× Denhardt's solution, and 100 μg ml^{-1} denatured salmon sperm DNA. A full-length 1.4 kb DNA probe of *OXI1* (At3g25250) was obtained through restriction digestion of *OXI1* cloned into the pUC2X35S plasmid with the enzymes *Pst*I and *Bam*HI. The *VSP1* (At5g24780) template of approximately 300 bp was amplified by PCR of genomic DNA with the primers 5′-CGGCATCCGTTCCAGCCGTC-3′ and 5′-CTAGAGAGGAGAGTGTCGTC-3′. The *PR-1* (At2g14610) probe was amplified from genomic DNA using primers previously described by Denby *et al.* (2005).

Results

OXI1 is necessary for full resistance to P. syringae

Given the requirement for OXI1 in the basal defence response to virulent *H. parasitica* (Rentel *et al.*, 2004), it was investigated whether OXI1 is required for defence against other virulent plant pathogens. The *oxi1* null mutant, wild type (Ws-2), and the *oxi1* mutant complemented with the wild-type *OXI1* gene (*oxi1+OXI1*) transgenic line were challenged with virulent *P. syringae* pv. *tomato* DC3000 (*Pst* DC3000). The *oxi1* mutant exhibited increased susceptibility at 2 and 3 d post-inoculation (dpi) compared with the wild type (Fig. 1A; see Supplementary Fig. S1A at *JXB* online). Importantly, the complemented line exhibited wild-type bacterial titres, demonstrating that the increased susceptibility phenotype of the *oxi1* mutation was due to the lack of *OXI1* expression (Fig. 1A; see Supplementary Fig. S1B at *JXB* online). OXI1 is therefore required for basal resistance against both an oomycete (*H. parasitica*) and a bacterial (*Pst* DC3000) biotrophic pathogen. Despite strong induction after infection with *Botrytis cinerea* (see Supplementary Fig. S2 at *JXB* online), o*xi1* mutants did not show altered susceptibility to this necrotrophic pathogen (data not shown).

It was also found that OXI1 is necessary for full resistance against an avirulent isolate of *P. syringae* which carries the *avrB* gene (*Pst* DC3000 *avrB*) (Fig. 1B; see Supplementary Fig. S1C at *JXB* online). Again, the complemented line contained bacterial titres similar to the wild type. The requirement for *OXI1* for full resistance was confirmed using an additional avirulent isolate of *P. syringae* (*Pst* DC3000 carrying *avrRpt2*) (see Supplementary Fig. S3 at *JXB* online). Hence, although defence against avirulent *H. parasitica* isolates is OXI1-independent (Rentel, 2002), *OXI1* is required for full resistance against both virulent and avirulent *P. syringae*.

Overexpression of OXI1 *results in increased susceptibility to biotrophic pathogens*

Having demonstrated that *oxi1* mutants are more susceptible to *P. syringae*, it was tested whether increased expression of *OXI1* could lead to enhanced resistance. Two independent overexpressor lines were generated; both drive *OXI1* expression from the *35S CaMV* promoter but one contains OXI1 fused to the reporter gene YFP. Both lines show increased *OXI1* expression at the mRNA level (Fig. 2). Surprisingly, these overexpressor lines displayed enhanced susceptibility to both virulent and avirulent isolates of *P. syringae* (Fig. 3A, B). Since both overexpressor lines showed the same phenotype, the increased susceptibility was not due to the position of the transgene or as a consequence of the YFP fusion. Due to this unexpected result, and as *oxi1* mutants show increased susceptibility to virulent *H. parasitica* (Rentel *et al.*, 2004), the susceptibility of these overexpressing lines to the virulent *H. parasitica* isolate Emco5 was tested (Fig. 3C). Again, the *35S::OXI1* overexpressor showed enhanced susceptibility (as seen by

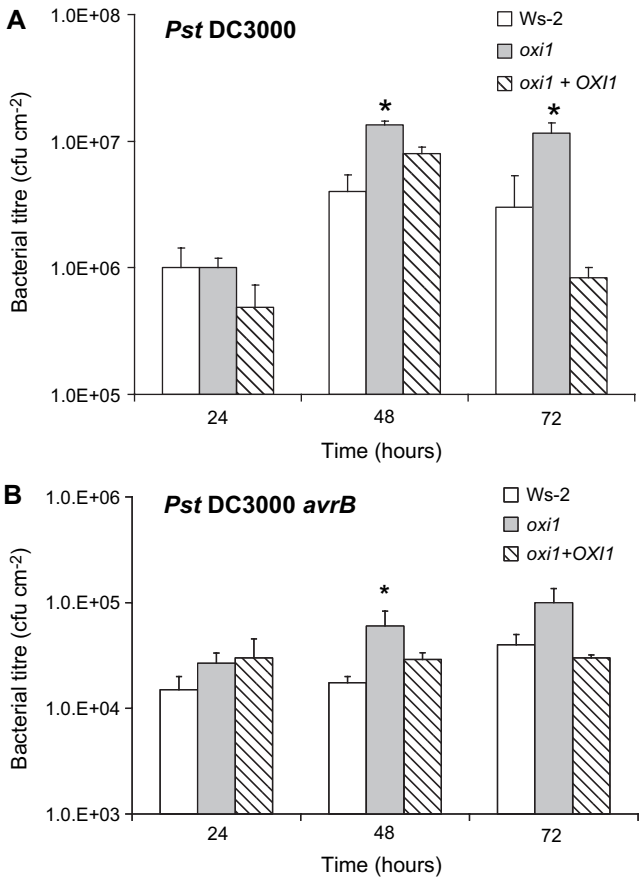

Fig. 1. The *oxi1* mutant exhibits increased susceptibility to both virulent and avirulent strains of *P. syringae*. Leaves of 3-week-old wild-type Ws-2, *oxi1* mutant, and the *oxi1* mutant line complemented with the wild-type *OXI1* gene (*oxi1+OXI1*) were pressure inoculated with either virulent *Pst* DC3000 (A) or avirulent *Pst* DC3000 harbouring the *avrB* (B) gene at 5×10^5 cfu ml^{-1} and bacterial titre determined. The bars represent the mean log bacterial titre expressed as cfu cm^{-2} at 24, 48, and 72 h post-infection ±SEM (n=3 biological replicates, each consisting of three leaf discs per replicate plant). An asterisk indicates a significant increase in pathogen growth compared to the wild type (Student's t test, P <0.05). Results shown are for one representative experiment of four.

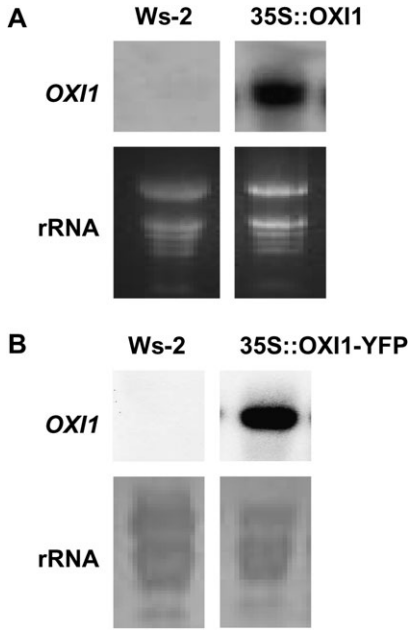

Fig. 2. Transgenic *Arabidopsis* lines overexpressing *OXI1* and *OXI1-YFP*. Northern analysis confirmed constitutive overexpression of *OXI1* (A) or *OXI1-YFP* (B) in transgenic *Arabidopsis* lines. Blots were probed with a full-length *OXI1* DNA fragment that recognizes wild-type *OXI1* and the larger *OXI1-YFP* transcript. Ethidium bromide- (A) or methylene blue- (B) stained rRNA was used as a loading control. Results shown are for one representative experiment of two.

increased sporulation) compared to the wild type. Sporulation in the *35S::OXI1-YFP* line was highly variable, hence, although the average susceptiblity was increased, the result was not statistically significant. From these results it was concluded that modulation of *OXI1* expression levels (either increased or knocked out) causes increased susceptibility to virulent and avirulent *P. syringae* as well as to virulent *H. parasitica* isolates.

Expression of two defence marker genes is uncompromised in the oxi1 *null mutant*

As OXI1 is required for resistance against at least two biotrophic pathogens, an attempt was made to establish a functional basis for this requirement. However, expression of the classic defence gene *PR-1* was not compromised in the *oxi1* mutant following infection with avirulent *P. syringae* (Fig. 4A; see Supplementary Fig. S4A at *JXB* online). Given that OXI1 is required for full activation of MPK3 amd MPK6 in response to ROS and cellulase treatment (Rentel *et al.*, 2004), it was investigated whether OXI1 regulates the expression of MPK6-dependent *Vegetative Storage Protein1* (*VSP1*) in response to pathogen challenge. *VSP1* was induced only 48 h after infection with virulent *P. syringae,* again this induction was not affected in the *oxi1* mutant (Fig. 4B; see Supplementary Fig. S4B at *JXB* online). As expected, *VSP1* was not induced in response to challenge with avirulent *P. syringae* in either wild-type or *oxi1* mutant plants (data not shown).

The oxidative burst mediates induction of OXI1 *expression*

Expression of *OXI1* is known to be induced in response to ROS (H_2O_2) and in cells adjacent to *H. parasitica* hyphae (Rentel *et al.*, 2004). As ROS production is one of the earliest plant responses to pathogen infection (Lamb and Dixon, 1997), it was investigated whether ROS accumulation was responsible for the induction of *OXI1* gene expression after *P. syringae* infection. After infection with either virulent or avirulent *P. syringae*, GUS expression driven by the *OXI1* promoter increased and was confined to the regions of ROS accumulation in the leaf (Fig. 5A). Two methods were used to reduce the rapid oxidative burst which occurs after avirulent *P. syringae* infection and to determine the effect on

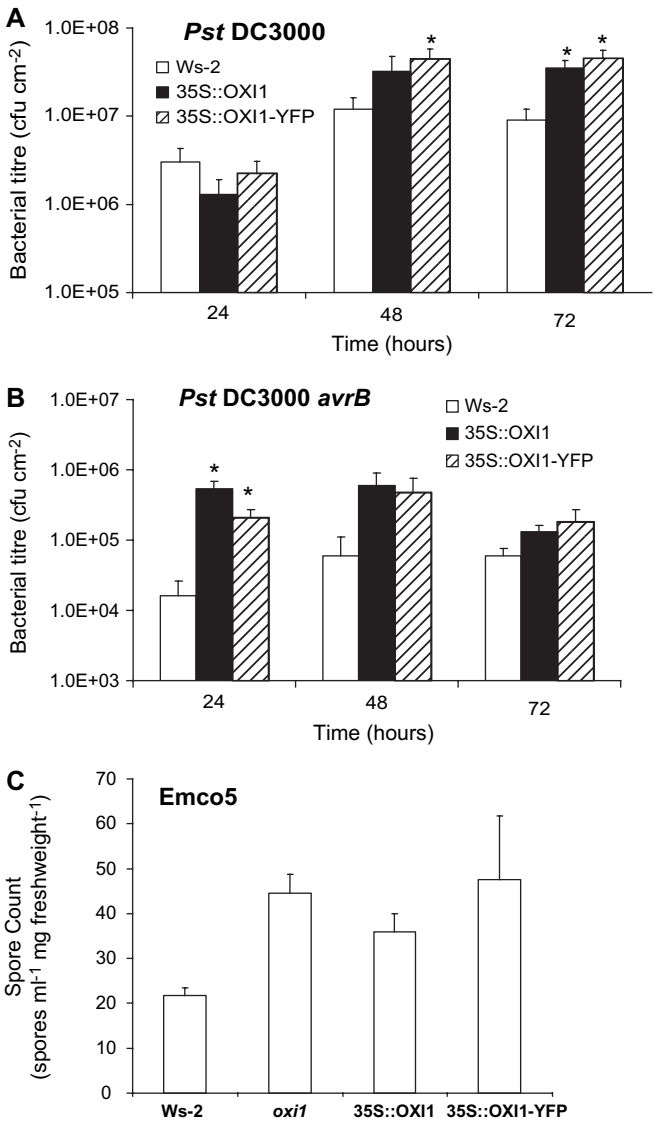

Fig. 3. Overexpression of *OXI1* increases susceptibility to the biotrophic pathogens *P. syringae* and *H. parasitica*. Analysis of bacterial titres in 3-week-old leaves of Ws-2, *oxi1*, *35S::OXI1*, and *35S::OXI1-YFP* plants pressure-inoculated with either 5×10^5 cfu ml^{-1} virulent *Pst* DC3000 (A) or avirulent *Pst* DC3000 *avrB* (B). The bars represent the mean log bacterial titre expressed as cfu cm^{-2} ±SEM (n=3 biological replicates, each consisting of three leaf discs per replicate plant). (C) Seven-day-old seedlings of genotypes Ws-2, *oxi1*, *35S::OXI1*, and *35S::OXI1-YFP* were sprayed with spores of the virulent *H. parasitica* strain Emco5 at a spore suspension of 5×10^4 spores ml^{-1}. Bars represent the average sporulation of four independent samples of pooled seedlings for each genotype, 7 d post-infection ±SEM. An asterisk indicates a significant increase in pathogen growth compared to the wild type (Student's *t* test, P <0.05). Results shown are for one representative experiment of four.

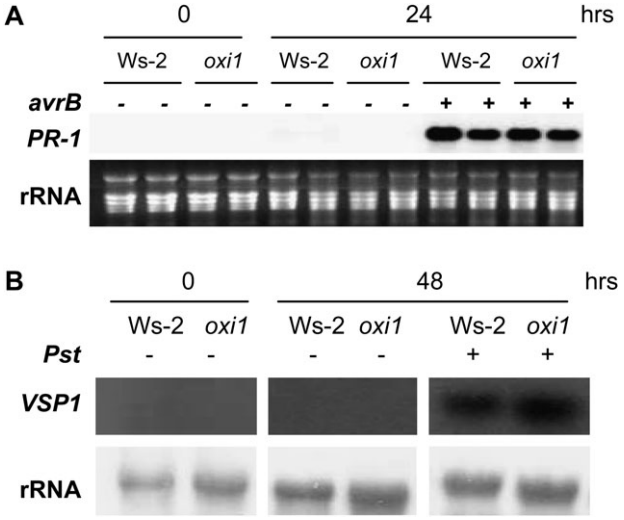

Fig. 4. Expression of defence associated genes in the *oxi1* mutant. Three-week-old Ws-2 and the *oxi1* mutant were pressure-inoculated with either 10 mM $MgCl_2$ alone (–) or 5×10^5 cfu ml^{-1} avirulent *Pst* DC3000 *avrB* (*avrB*) or virulent *Pst* DC3000 (*Pst*) in a 10 mM $MgCl_2$ suspension. Leaves were harvested before the start of the experiment (0) or at various time points post-infection as indicated. Expression of *PR-1* (A) and *VSP1* (B) was assessed via Northern analysis. Ethidium bromide- (A) and methylene blue- (B) stained RNA were used as loading controls. Results shown are for one representative experiment of two.

OXI1 expression. As expected, an *atrbohD* mutant failed to accumulate H_2O_2 during infection with avirulent *Pst* DC3000 *avrB* (Fig. 5B). *OXI1* expression in this mutant background was reduced compared to the wild-type control (Fig. 5C), suggesting that ROS generated through NADPH oxidase is at least partly responsible for the induction of *OXI1* during ETI. This conclusion was strengthened by reduced GUS activity in leaves of *OXI1::GUS* plants co-infiltrated with *Pst* DC3000 *avrB* and 10 μM diphenylene iodinium (DPI), a chemical inhibitor of NADPH oxidase, compared to leaves infiltrated with *Pst* DC3000 *avrB* alone (Fig. 5D; see Supplementary Fig. S5 at *JXB* online).

Discussion

Pathogen-induced *OXI1* expression is the result of ROS accumulation, produced at least in part, via the AtrbohD NADPH-oxidase mechanism (Fig. 5) and OXI1 clearly contributes to both basal and effector-triggered resistance to the bacterial pathogen *P. syringae* (Fig. 1). It is thought that the regulation of PTI and ETI resistance responses overlaps considerably, with ETI being an accelerated and amplified PTI response (Jones and Dangl, 2006). Large-scale expression profiling provided evidence that ETI is qualitatively similar to PTI as the expression profiles, as well as the level of induction of genes, during the early stages of infection with *P. syringae* pv. *maculicola* (*Psm*) harbouring the avirulence *avrRpt2* gene were similar to those produced during the late stages of infection with virulent *Psm* (Tao *et al.*, 2003). Furthermore, *Arabidopsis* mutant analysis has identified many molecular components in the defence signalling network involved in both PTI and ETI. For example, a mutation in the *ENHANCED*

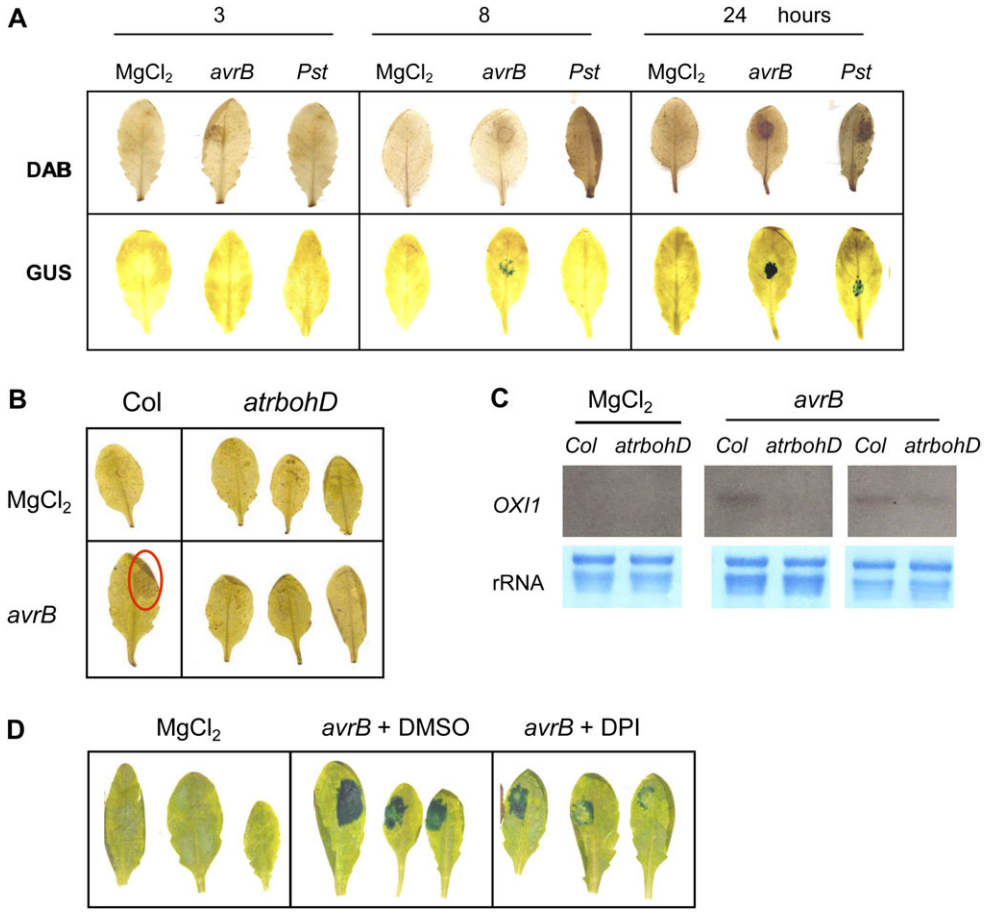

Fig. 5. *OXI1* expression correlates with the oxidative burst following pathogen infection and is at least partially dependent on NADPH oxidase. (A) A small area of leaf tissue from 4-week-old *OXI1::GUS* transgenic plants was pressure-inoculated with either 10 mM $MgCl_2$, avirulent *Pst* DC3000 *avrB* (*avrB*) or virulent *Pst* DC3000 (*Pst*) suspension in 10 mM $MgCl_2$ at 5×10^6 cfu ml^{-1}. At 3, 8, and 24 h post-infection leaves were excised and stained with DAB for the presence of an oxidative burst (forms a reddish-brown precipitate with H_2O_2) or stained for GUS activity. Three leaves were analysed per treatment per time point; one representative leaf of each sample is shown. Both the oxidative burst and *OXI1* expression occurred within the infected area. (B) A small area of leaves from 4-week-old *atrbohD* mutant and wild-type Col-0 plants were pressure-inoculated with either 10 mM $MgCl_2$ or avirulent *Pst* DC3000 *avrB* (*avrB*) suspension in 10 mM $MgCl_2$ at 5×10^6 cfu ml^{-1}. DAB staining at 6 h post-infection demonstrated the lack of the oxidative burst in the *atrbohD* mutant. A representative of three leaves per treatment of Col is shown and the red circle indicates the infected area exhibiting an oxidative burst. (C) Entire leaves of 4-week-old *atrbohD* mutants and wild-type Col were pressure-inoculated with either 10 mM $MgCl_2$ or avirulent *Pst* DC3000 *avrB* (*avrB*) suspension in 10 mM $MgCl_2$ at 5×10^6 cfu ml^{-1}. A minimum of four leaves were harvested at 8 h post-inoculation for each sample. Duplicate samples for each genotype are shown. Northern blots were probed with a full-length *OXI1* DNA fragment. Methylene blue-stained RNA was used as a loading and transfer control. (D) A small area of leaves from 4-week-old *OXI1::GUS* transgenic plants was pressure-inoculated with either 10 mM $MgCl_2$ or avirulent *Pst* DC3000 *avrB* suspension in 10 mM $MgCl_2$ containing either 0.1% (v/v) DMSO (*avrB*+DMSO) or 10 μM DPI (*avrB*+DPI) at 5×10^6 cfu ml^{-1}. DPI is a chemical inhibitor of NADPH oxidase and co-infiltration with *avrB*+DMSO was used as a control for the DPI treatment. GUS activity was visualized 24 h post-infection and showed a reduction with DPI treatment. In all cases, results are shown from one representative experiment of three.

DISEASE SUSCEPTIBILITY1 (*EDS1*) gene results in *eds1* mutants being more susceptible to both virulent and avirulent isolates of *H. parasitica* (Parker *et al.*, 1996). Similarly, the *Arabidopsis* mutants *eds5, npr1*, *sid2*, and *pad4* are compromised in their resistance responses to both virulent and avirulent isolates of *H. parasitica* and/or *P. syringae* (Cao *et al.*, 1994; Zhou *et al.*, 1998; Nawrath and Métraux, 1999). Silencing of MPK6, a component of the *Arabidopsis* MAPK cascade induced by flagellin during PTI, also leads to enhanced susceptibility to virulent and avirulent isolates of *P. syringae* (Menke *et al.*, 2004). Our data indicate that OXI1 represents another shared component between PTI and ETI since the *oxi1* mutant exhibits enhanced bacterial titres compared to the wild type following infection with both virulent and avirulent *P. syringae.* OXI1 is likely to trigger phosphorylation events that result in the activation of defence responses that serve to restrict or slow the process of pathogen growth.

In contrast to infection by *P. syringae*, a role for OXI1 could only be discerned in the defence against a virulent

H. parasitica isolate and not the avirulent isolate Emoy2 (Rentel *et al.*, 2004). Only one avirulent isolate of *H. parasitica* has been tested and it is possible that OXI1 may play a role in ETI against isolates with different effector complements. Given that effectors themselves target different components of the defence system (which are then unavailable for signalling), it is unlikely that ETI signalling will be identical in response to all effectors.

While infection with the necrotrophic pathogen *B. cinerea* resulted in increased expression of *OXI1* in *Arabidopsis* (see Supplementary Fig. S1 at *JXB* online), lack of OXI1 did not increase susceptiblity compared with the wild type (data not shown). Induction of *OXI1* in response to *B. cinerea* appears to be a consequence of ROS accumulation during this interaction, without an active role in defence. Hence our results currently limit the role of OXI1 to disease resistance against biotrophic pathogens.

A surprising feature of OXI1 is that reduced expression and overexpression of *OXI1* both led to enhanced susceptibility to biotrophic pathogens (Fig. 3). It is unlikely that a single kinase plays both a positive and a negative role during the same defence response. Hence it is proposed that the level of OXI1 protein is crucial for the appropriate signalling, and modulation of these levels (either higher or lower) disrupts OXI1 function. As transcript levels do not necessarily correlate with protein levels, it is possible that the OXI1 overexpression lines either have lower or higher OXI1 protein levels compared to wild type since these lines mirror the loss of function mutant. In the first instance, if the protein levels are actually reduced in the overexpression lines relative to the wild type, it could be that prolonged expression of OXI1 protein from a constitutive promoter (35S) might lead to enhanced activation of pathways naturally present to regulate the OXI1 protein negatively, i.e. by protein degradation. Alternatively, it would be more orthodox and parsimonious to assume that protein levels are higher in the overexpression lines than in the wild type. It is not uncommon for plants overexpressing proteins to show the same phenotypes as loss of function mutants, for example, FIP1 and EBS, also in *Arabidopsis* (Pineiro *et al.*, 2003; Chen *et al.*, 2007). In these cases the perceived wisdom is that these proteins operate in complexes in which the stoichiometry is crucial and regulated by the levels of protein expression. Therefore, both under- or over-expression would lead to suboptimal complex formation, leading to reduced function. Therefore the absolute level of abundance of such components is crucial and OXI1 may be one such component. Furthermore, OXI1 levels appear to be tightly controlled *in planta*; *35S::OXI1-YFP* lines show low levels of YFP protein compared with lines expressing YFP-aequorin protein in single root cell types, and treatment of seedlings with the proteasome inhibitor MG132 for 1 h results in dramatically increased protein levels indicating a short half-life (data not shown). This rapid turnover of OXI1 protein together with the importance of timing in pathogen responses presents another scenario whereby the key factor could also be one of appropriate timing. For example, having either OXI1 protein or transcript already present when plants are challenged with pathogen may be detrimental to establishing an optimal defence response.

Despite the demonstration of MPK6 as a downstream component of OXI1 in response to H_2O_2 and cellulase treatment (Rentel *et al.*, 2004) and the fact that the defence phenotype of *mpk6* silenced *Arabidopsis* mutants resembles that of *oxi1* (Menke *et al.*, 2004), MPK6-dependent expression of *VSP1* during PTI appears to be independent of OXI1 (Fig. 4B). Similarly, despite the importance of SA signalling to disease resistance against biotrophic pathogens, OXI1 was not required for expression of the SA marker gene *PR1* (Fig. 4A) or development of systemic acquired resistance (see Supplementary Fig. S6 at *JXB* online). OXI1 interacts directly with, and can phosphorylate, the Ser/Thr protein kinase PTI1-2 (Anthony *et al.*, 2006). PTI1-2 is activated in response to various stress treatments and is dependent on OXI1 for its activation in response to flagellin and H_2O_2 (Anthony *et al.*, 2006). Given the homology between PTI1-2 and the tomato Pto kinase, which confers resistance to avirulent *P. syringae* carrying the *avrPto* gene (Zhou *et al.*, 1995), it is tempting to speculate that OXI1 promotes defence against *P. syringae* through the activation of PTI1-2. However, no targets of PTI1-2 have been identified and, unlike Pto, OXI1 is likely to function downstream of ROS. Hence, the identification of additional direct targets of OXI1 will be vital in elucidating the function of this ROS-responsive kinase during *Arabidopsis-Pseudomonas/Hyaloperonospora* interactions and in addressing how specificity of ROS signalling is achieved.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. The *oxi1* null mutant is more susceptible to both virulent and avirulent *P. syringae* (A, C) and this phenotype of the null mutant is rescued in the complemented line which exhibits wild-type bacterial titres when challenged with *P. syringae* (B, C).

Supplementary Fig. S2. *OXI1* expression is induced following infection of *Arabidopsis* leaves with the necrotrophic pathogen *Botrytis cinerea*.

Supplementary Fig. S3. The *oxi1* null mutant is more susceptible than the wild type to the avirulent strain *Pst* DC3000 *avrRpt2*.

Supplementary Fig. S4. Expression of *PR-1* and *VSP1* is unaffected in the *oxi1* null mutant in response to pathogen challenge.

Supplementary Fig. S5. DPI treatment alone has no effect on *OXI1::GUS* expression.

Supplementary Fig. S6. The *oxi1* mutation does not affect the development of systemic acquired resistance.

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